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3. Full name, address and postcode of the or of each applicant (underline all surnames)

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Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

UNITED KINGDOM

4. Title of the invention

Excretal Reporter Systems

PE
X
7
1

5. Name of your agent (if you have one)

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Abstract

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I/We request the grant of a patent on the basis of this application.

Signature

Hansen Goddard Foote

Date

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12. Name and daytime telephone number of person to contact in the United Kingdom

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Excretable Reporter Systems

The present invention relates to reporter systems comprising a reporter gene encoding a reporter protein that is secretable from cells in which it is produced or expressed either 5 *in vitro* or *in vivo* and excretable from whole animals comprising such systems. The reporter system is for the detection of gene activation events or biochemical changes related to, or that occur, as a result of altered metabolic or disease status or toxicological stress both for use, particularly but not exclusively, in toxicological screening. The present invention also provides methods of making such systems and 10 uses thereof.

Background to the Invention

Genes encode proteins. It is estimated that there are at least 3×10^4 genes in the 15 vertebrate genome but for a given cell only a subset of the total number of genes is active, with the subset differing between cells of different types and between different stages of development and differentiation (Cho & Campbell *Trends Genet.* 16 409-415 (2000); Velculescu *et al* *Trends Genet.* 16 423-425 (2000)). The DNA regulatory elements associated with each gene governs the decision as to which genes are active 20 and which are not. Although comprising a number of defined elements these DNA sequences are collectively termed promoters (Tjian & Maniatis *Cell* 77 5-8 (1994); Bonifer, *Trends Genet.* 16 310-315 (2000); Martin, *Trends Genet* 17 444-448 (2001)).

Gene activation occurs primarily at the transcriptional level. Transcriptional activity of 25 a gene may be measured by a variety of approaches including RNA polymerase activity, mRNA abundance or protein production (Takano *et al.*, 2002). These approaches are limited in that they require development of an assay suitable to each individual mRNA or protein product. To facilitate comparison of different promoters, rather than assaying individual gene products, reporter genes are often used (Sun *et al* 30 *Gene Ther.* 8 1572-1579 (2001); Franco *et al* *Eur. J. Morphol.* 39 169-191 (2001);

Hadjantonakis & Nagy, *Histochem. Cell. Biol.* 115 49-58 (2001); Gorman *Mol. Cell. Biol.* 2 1044-1051 (1982); Barash and Reichenstein, 2002; Zhang et al., 2001.).

5 The product (mRNA or protein) of a reporter gene allows an assessment of the transcriptional activity of a particular gene and can be used to distinguish cells, tissues or organisms in which the event has occurred from those in which it has not. On the whole, reporter genes are foreign to the host cell or organism, allowing their activity to be easily distinguished from the activity of endogenous genes. Alternatively the reporter may be marked or tagged so as to make it distinct from host genes.

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Reporter genes are linked to the test promoter, enabling activity of the promoter gene to be determined by detecting the presence of the reporter gene product. Therefore, the main prerequisite for a reporter gene product is that it is easy to detect and quantify. In some cases, but not all, the reporter gene has enzymatic activity that catalyses the 15 conversion of a substrate into a measurable product.

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A classical example is the bacterial chloramphenicol acetyl transferase (CAT) gene. CAT activity can be measured in cell extracts as conversion of added non-acetylated chloramphenicol to the acetylated form of chloramphenicol by chromatography (Gorman *Mol. Cell. Biol.* 2 1044-1051 (1982)). Further examples of enzymatic reporters include alkaline phosphatase, β -galactosidase, thymidine kinase, neomycin resistance and growth hormone. Similar strategies enable the use of the firefly 25 luciferase gene as a reporter. However, in this instance it is the light produced by bioluminescence of the luciferin substrate that is measured.

25

Some reporters also benefit from the visual detection assays that allow *in situ* analysis of reporter activity. A frequently used example would be β -galactosidase (Lac Z), where the addition of an artificial substrate, X-gal, enables reporter activity to be detected by the appearance of blue coloration in the sample. As it is accumulative it 30 effectively provides an historical record of its induction. This is particularly useful for

measuring transient responses where a promoter is activated for only a short time before being rapidly inactivated. This reporter has been successfully used both in cultured cells and *in vivo* (Campbell *et al* *J. Cell. Biol.* **109** 2619-2625 (1996)), though its suitability for *in vivo* use has been questioned in some reports (Chevalier-Mariette *et al.*, *Genome Biol.* **4** R53 (2003); Sanchez-Ramnos *et al* *Cell Transplant.* **9** 657-667 (2000); Montoliu *et al* *Transgenic Res.* **9** 237-239 (2000); Cohen-Tannoudji *et al* *Transgenic Res.* **9** 233-235 (2000)). It has been demonstrated that Lac Z, in combination with fluorescent substrates can enable the sorting of cells that express the reporter by use of a fluorescence-activated cell sorter (FACS) (Fiering *et al* *Cytometry* **12** 291-301 (1991)).

In other systems, the reporter product itself is directly detected, removing the need for a substrate. Green fluorescent protein has become one of the most commonly used examples of this category of reporter (Ikawa *et al* *Curr. Top. Dev. Biol.* **44** 1-20 (1997)). This autofluorescing protein was derived from the bioluminescent jellyfish *Aequoria victoria*. Several colour spectral variants of this reporter have been developed (Hadjantonakis & Nagy, *Histochem. Cell. Biol.* **115** 49-58 (2001)).

Recently reporter systems based on energy emission systems have been developed. These include single photon emission computed tomography (SPECT) and positron emission tomography (PET) though these require the introduction of a radiolabelled isotope probe in to the host cell or animal that is then modified by the target reporter gene. For example the PET system measures reporter sequestering of the positron-emitting probe (Sun *et al* *Gene Ther.* **8** 1572-1579 (2001)).

Many tried and tested reporter systems have been developed but nevertheless they share certain limitations. Those based on prokaryote genes often suffer poor expression in transgenic mammals (Montoliu *et al* *Transgenic Res.* **9** 237-238 (2000); Cohen-Tannoudji *et al* *Transgenic Res.* **9** 233-235 (2000)). Furthermore the presence of prokaryote DNA sequences has been implicated in the suppression of expression from

adjacent eukaryote transgenes as have the presence of intronless, cDNA based eukaryote gene sequences (Clark et al., 1997).

Most of the current reporters, whilst useful for monitoring expression under certain 5 circumstances, have certain limitations. Many accumulate in cells and are not useful for monitoring changes in promoter activation over time. Perhaps more importantly detection of expression necessitates the fixing of cultured cells or the sacrifice of transgenic animals, thus limiting reporters to invasive detection strategies. There are a few exceptions; These include the use of growth hormone (Bchini *et al* *Endocrinology* 10 128 539-546 (1991)). However its high biological activity effectively limit its widespread applicability. GFP has been detected in whole animals and though possessing relatively low biological activity its use has so far been limited to neonatal and nude mice in which both internal tissue and dermal fluorescence are more readily observed. In addition there has been a report that GFP is cytotoxic (Liu *et al* *Biochem. Biophys. Res. Comm.* 260 712-717 (1999)). Although reporter systems based on tomography allow monitoring of reporter expression in internal tissues they require addition of exogenously added substrates that could potentially confound results by influencing expression of the reporter. Additionally they can lack the sensitivity required for quantitative analysis of reporter expression.

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There is therefore a need for a reporter system that overcomes some or all of these limitations. In the first instance the reporter should be secreted (from the cell in which it is expressed or produced) and preferably excreted (from the whole animal) so that advantageously the system is non-invasive inasmuch as its detection does not involve 25 addition of an external substrate or sacrifice of transgenic animals. In addition, the system should be biologically neutral with regard to the test expression system so that no phenotypic effects either confound readout from the system or affect the health of the transgenic animal.

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A system satisfying such requirements has now been found. The non-invasive reporters of the present invention comprise characteristics which favour secretion from

the cell where it is produced or expressed and excretion of the gene product or metabolite into a body fluid, relative stability and distinction from native molecules.

Statement of the Invention

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In its broadest aspect the present invention provides a biological reporter system that permits non-invasive measurement of biochemical changes arising as result of toxic insult/stress, constitutive or induced disease states and/or altered metabolic status.

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According to a first aspect of the invention, there is provided a nucleic acid construct comprising a nucleic acid sequence comprising a reporter gene encoding a reporter protein that is secretable as a protein or product from a cell where it is expressed or produced and that is excretable from a whole animal.

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Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

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Reference herein to protein or product is intended to include: protein complexes or fragments; enzymes; enzymatic products or conjugates; primary, secondary or further metabolites and/or salts thereof; non-biological products that are released by direct or secondary effects on the expression of the reporter gene product; hormones or antibodies.

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Throughout the present specification the reporter system proteins of the present invention are conveniently referred to as a secreted/excreted proteins or products. It will be appreciated by the skilled man that this term relates to the reporter protein or product being firstly secreted from a cell where it is either expressed or produced and subsequently being excreted into a body fluid from where it may be measured or monitored or assayed.

Preferably, the secreted/excreted protein/product may be produced as a result of modulated gene transcription.

Alternatively, the secreted/excreted reporter protein/product may be produced and secreted/excreted as a result of increased reporter translation for example as a result of increased stability or decreased turnover of m RNA.

As a yet further alternative, the reporter protein/product may be as the result of post translational modification such as increased reporter stability through removal of polyubiquination or alternatively the reporter protein/product may be as the result of accumulation or excretion of small molecule metabolites.

We have found surprisingly that the activity of SEAP, a secreted version of alkaline phosphatase, can be induced both *in vitro* and *in vivo* and that it is excreted in body fluids such as the blood and urine of transgenic animals. Accordingly, SEAP is an example of a secreted/excreted reporter protein of the present invention. However it will be apparent that other reporter proteins having similar characteristics described herein after will also be suitable for the present invention.

Therefore in one embodiment of the invention the nucleic acid construct comprises a nucleic acid sequence comprising a reporter gene encoding the SEAP reporter protein that is secretable as a protein or product from a cell where it is expressed or produced and that is excretable from a whole animal.

In a further embodiment of the invention the reporter protein or product may comprise a peptide tag such as an epitope tag or a tag which may possess enzymatic activity to convert a substrate to a form that is readily detectable by an assay. This embodiment of the invention advantageously provides for multiple reporter systems in a single cell or single animal.

It will be appreciated that the position of the peptide tag may be at the amino terminal or carboxy terminal or inserted internally with respect to the amino acid sequence of the reporter, and that in the instance of the tag being an epitope tag that it is recognised by its cognate antibody irrespective of its location in the reporter protein.

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The epitope tag may be a defined amino acid sequence from a protein with a fully characterised cognate antibody. The skilled person can select such epitopes based on sequences identified as possessing antigenic properties. In certain embodiments of the invention the epitope tag may be the amino acid sequence below from the c-myc oncogene (Evans *et al* *Mol. Cell. Biol.* 5 3610-3616 (1985)):

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-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-

or it may be the amino acid sequence from the simian virus V5 protein (Southern *et al* *J. Gen. Virol.* 72 1551-1557 (1991)), shown below:

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-Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr-

In certain embodiments of the invention, the epitope may be selected from but not limited to the c-myc and V5 proteins.

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In some instances the peptide tag may possess enzymatic activity that converts a substrate to a form that is readily detectable by an assay, the tag may have kinase activity specifying phosphorylation of another protein or peptide substrate that could be added to the secreted or excreted analyte along with a phosphate group donor.

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Detection could be achieved using an immunological assay based on detection by an antibody specifically recognising the phosphorylated version of the tagged reporter protein. Such assays include ELISA, Western blot, RIA and fluorescence polarisation. Alternatively the use a released labelled product for example, phosphate radiolabelled with an isotope of phosphorous such as ^{32}P or ^{33}P , which could be measured by fluorometric, radioactive or colorimetric means. Other enzymic modifications include for example acetylation, sulphation and glycosylation.

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Other embodiments of this aspect could include, for example site of interaction with protein other than antibody e.g. lectin binding site, or modification of tag by e.g. addition of amino acid multimer such as polylysine; or incorporation of a fluorochrome.

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According to the various embodiments of this aspect of the invention, the reporter gene may be associated with a promoter. The promoter will preferably be of mammalian origin, but also may be from a non-mammalian animal, plant, yeast or bacteria. The promoter may be selected from but is not limited to promoter elements of 10 the following inducible genes:

whose expression is modified in response to disturbances in the homeostatic state of DNA in the cell. These disturbances may include chemical alteration of nucleic acids or precursor nucleotides, inhibition of DNA synthesis and inhibition of DNA replication or damage to DNA. The sequence can be selected from but not limited to the group consisting of c-myc (Hoffman *et al* *Oncogene* **21** 3414-3421), p21/WAF-1 (El-Diery *Curr. Top. Microbiol. Immunol.* **227** 121-137 (1998); El-Diery *Cell Death Differ.* **8** 1066-1075 (2001); Dotto *Biochim. Biophys. Acta* **1471** 43-56 (2000)), MDM2 (Alarcon-15 Vargas & Ronai *Carcinogenesis* **23** 541-547 (2002); Deb & Front *Bioscience* **7** 235-243 (2002)), Gadd45 (Sheikh *et al* *Biochem. Pharmacol.* **59** 43-45 (2000)), FasL (Wajant *Science* **296** 1635-1636 (2002)), GAHSP40 (Hamajima *et al* *J. Cell. Biol.* **84** 401-407 (2002)); TRAIL-R2/DR5 (Wu *et al* *Adv. Exp. Med. Biol.* **465** 143-151 (2000); El-Diery *Cell Death Differ.* **8** 1066-1075 (2001)), BTG2/PC3 (Tirone *et al* *J. Cell. Physiol.* **187** 155-165 (2001)); 20 25

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whose transcription is modified in response to oxidative stress or hypoxia. The sequence can be selected from but not limited to the group consisting of MnSOD and/or CuZnSOD (Halliwell *Free Radic. Res.* **31** 261-272 (1999); Gutteridge & Halliwell *Ann. NY Acad. Sci.* **899** 136-147 (2000)), I B (Ghosh

& Karin *Cell* **109** Suppl., S81-96 (2002)), ATF4 (Hai & Hartman *Gene* **273** 1-11 (2001)), xanthine oxidase (Pristos *Chem. Biol. Interact.* **129** 195-208 (2000)), COX2 (Hinz & Brune *J. Pharmacol. Exp. Ther.* **300** 376-375 (2002)), iNOS (Alderton *et al* *Biochem. J.* **357** 593-615 (2001)), Ets-2 (Bartel *et al* *Oncogene* **19** 6443-6454 (2000)), FasL/CD95L (Wajant *Science* **296** 1635-1636 (2002)), γ GCS (Lu *Curr. Top. Cell. Regul.* **36** 95-116 (2000); Soltaninassab *et al* *J. Cell. Physiol.* **182** 163-170 (2000)), ORP150 (Ozawa *et al* *Cancer Res.* **61** 4206-4213 (2001); Ozawa *et al* *J. Biol. Chem.* **274** 6397-6404 (1999)).

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whose expression is modified in response to hepatotoxic stress. The sequence can be selected from but not limited to the group consisting of Lrg-21 (Drysdale *et al* *Mol. Immunol.* **33** 989-998 (1996)), SOCS-2 and/or SOCS-3 (Tollet-Egnell *et al* *Endocrinol.* **140** 3693-3704 (1999)), PAI-1 (Fink *et al* *Cell. Physiol. Biochem.* **11** 105-114 (2001)), GBP28/adiponectin (Yoda-Murakami *et al* *Biochem. Biophys. Res. Commun.* **285** 372-377 (2001)), α -1 acid glycoprotein (Komori *et al* *Biochem Pharmacol.* **62** 1391-1397 (2001)), metallothioneine I (Palmiter *et al* *Mol. Cell. Biol.* **13** 5266-5275 (1993)), metallothioneine II (Schlager & Hart *App. Toxicol.* **20** 395-405 (2000)), ATF3 (Hai & Hartman *Gene* **273** 1-11 (2001)), IGFbp-3 (Popovici *et al* *J. Clin. Endocrinol. Metab.* **86** 2653-2639 (2001)), VDGF (Ido *et al* *Cancer Res.* **61** 3016-3021 (2001)) and HIF1 α (Tacchini *et al* *Biochem. Pharmacol.* **63** 139-148 (2002)).

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whose expression is modified in response to a pro-apoptotic stimulus. The sequence can be selected from but not limited to the group consisting of Gadd 34 (Hollander *et al* *J. Biol. Chem.* **272** 13731-13737 (1997)), GAHSP40 (Hamajima *et al* *J. Cell. Biol.* **84** 401-407 (2002)), TRAIL-R2/DR5 (Wu *et al* *Adv. Exp. Med. Biol.* **465** 143-151 (2000); El-Diery *Cell Death Differ.* **8** 1066-1075 (2001)), c-fos (Teng *Int. Rev. Cytol.* **197** 137-202 (2000)),

CHOP/Gadd153 (Talukder *et al* *Oncogene* **21** 4280-4300 (2002)), APAF-1 (Cecconi & Gruss *Cell. Mol. Life Sci.* **5** 1688-1698 (2001)), Gadd45 (Sheikh *et al* *Biochem. Pharmacol.* **59** 43-45 (2000)), BTG2/PC3 (Tirone *J. Cell. Physiol.* **187** 155-165 (2001)), Peg3/Pwl (Relaix *et al* *Proc. Nat'l Acad. Sci. USA* **97** 2105-2110 (2000)), Sial1a (Maeda *et al* *FEBS Lett.* **512** 223-226 (2002)), S29 ribosomal protein (Khanna *et al* *Biochem. Biophys. Res. Commun.* **277** 476-486 (2000)), FasL/CD95L (Wajant *Science* **296** 1635-1636 (2002)), tissue transglutaminase (Chen & Mehta *Int. J. Cell. Biol.* **31** 817-836 (1999)), GRP78 (Rao *et al* *FEBS Lett.* **514** 122-128 (2002)), Nur77/NGFI-B (Winoto *Int. Arch. Allergy Immunol.* **105** 344-346 (1994)), CyclophilinD (Andreeva *et al* *Int. J. Exp. Pathol.* **80** 305-315 (1999)), p73 (Yang *et al* *Trends Genet.* **18** 90-95 (2002)) and Bak (Lutz *Biochem. Soc. Trans.* **28** 51-56 (2000)).

whose expression is modified in response to the administration of chemicals or drugs or other xenobiotic agents. The sequence can be selected from but not limited to the list comprised of xenobiotic metabolising cytochrome p450 enzymes from the 2A, 2B, 2C, 2D, 2E, 2S, 3A, 4A and 4B gene families (Smith *et al* *Xenobiotica* **28** 1129-1165 (1998); Honkaski & Negishi *J. Biochem. Mol. Toxicol.* **12** 3-9 (1998); Raucy *et al* *J. Pharmacol. Exp. Ther.* **302** 475-482 (2002); Quattrochi & Guzelian *Drug Metab. Dispos.* **29** 615-622 (2001)).

whose expression is modified in response to disease states either natural, modelled or induced. These diseases can be selected from but not limited to the list comprised of obesity, compromised immunity, degenerative neurological disorders, cancer, cardiovascular, inflammatory diseases, genetic diseases or metabolic disorders.

The promoter element may comprise a contiguous "wild-type" sequence or it may be a synthetic promoter sequence comprised of a minimal eukaryote consensus promoter

operatively linked to one or more sequence elements known to confer transcriptional inducibility in response to specific stimulus. A minimal eukaryotic consensus promoter is one that will direct transcription by eukaryotic polymerases only if associated with functional promoter elements or transcription factor binding sites. An 5 example of which is the PhCMV*-1 (Furth *et al* *Proc. Nat'l Acad. Sci. USA* **91** 9302-9306 (1994)). Sequence elements known to confer transcriptional induction in response to specific stimulus include promoter elements (Montoliu *et al* *Proc. Nat'l Acad. Sci. USA* **92** 4244-4248 (1995)) or transcription factor binding sites; these will be chosen from but are not limited to the list comprising the aryl hydrocarbon (Ah)/Ah 10 nuclear translocator (ARNT) receptor response element, the antioxidant response element (ARE), the xenobiotic response element (XRE).

According to a further aspect of the invention there is provided a nucleic acid construct comprising a stress inducible promoter operatively isolated from a nucleic acid 15 sequence encoding a reporter protein that is secretable from a cell where it is expressed as a protein or product and that is excreted from a whole animal, said sequence being flanked by nucleic acid sequences recognised by a site specific recombinase, or by insertion such that it is inverted with respect to the transcription unit encoding a secreted/excreted reporter protein. The recombinase recognition sites are arranged in 20 such a way that the isolator sequence is deleted or the inverted promoter's orientation is reversed in the presence of the recombinase. The construct also comprises a nucleic acid sequence comprising a tissue specific promoter operatively linked to a gene encoding the coding sequence for the site specific recombinase.

This aspect allows for detecting reporter transgene induction in specified tissues only. 25 By controlling the appropriate recombinase expression using a tissue specific promoter, the inducible transgene will only be viable in those tissues in which the promoter is active. For example, by driving recombinase activity from a liver specific promoter, only the liver will contain re-arranged reporter construct, and hence will be the only tissue in which reporter induction can occur.

The recombination event producing an active reporter transcription unit may therefore only take place in tissues where the recombinase is expressed. In this way the reporter may only be expressed in specified tissue types where expression of the recombinase results in a functional transcription unit comprised of the inducible promoter linked to 5 the promoter. Site specific recombinase systems known to perform such a function include the bacteriophage P1 cre-lox and the bacterial FLP systems. The site specific recombinase sequences may therefore be two *loxP* sites of bacteriophage P1.

The use of site specific recombination systems to generate precisely defined deletions in 10 cultured mammalian cells has been demonstrated. Gu *et al.* (*Cell* 73 1155-1164 (1993)) describe how a deletion in the immunoglobulin switch region in mouse ES cells was generated between two copies of the bacteriophage P1 *loxP* site by transient expression of the Cre site-specific recombinase, leaving a single *loxP* site. Similarly, yeast FLP recombinase has been used to precisely delete a selectable marker defined by 15 recombinase target sites in mouse erythroleukemia cells (Fiering *et al.*, *Proc. Nat'l. Acad. Sci. USA* 90 8469-8473 (1993)). The Cre *lox* system is exemplified below, but other site-specific recombinase systems could be used.

A construct used in the Cre *lox* system will usually have the following three functional 20 elements:

1. The expression cassette;
2. A negative selectable marker (e.g. Herpes simplex virus thymidine kinase (TK) gene) expressed under the control of a ubiquitously expressed promoter (e.g. phosphoglycerate kinase (Soriano *et al.*, *Cell* 64 693-702 (1991)); and
3. Two copies of the bacteriophage P1 site specific recombination site *loxP* (Baubonis *et al.*, *Nuc. Acids. Res.* 21 2025-2029 (1993)) located at either end of 30 the DNA fragment.

This construct can be eliminated from host cells or cell lines containing it by means of site specific recombination between the two *loxP* sites mediated by Cre recombinase protein which can be introduced into the cells by lipofection (Baubonis *et al.*, *Nuc. Acids Res.* **21** 2025-2029 (1993)). Cells which have deleted DNA between the two *loxP* sites 5 are selected for loss of the TK gene (or other negative selectable marker) by growth in medium containing the appropriate drug (ganciclovir in the case of TK).

According to the further aspect of the invention there is provided a host cell transfected with at least one nucleic acid construct according to any one of the 10 previous aspects of the invention. The cell type is preferably of human or non-human mammalian origin but may also be of other animal, plant, yeast or bacterial origin.

It will be appreciated that the host cell may be transfected with a plurality of reporter systems according to the present invention.

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According to a yet further aspect of the invention, there is provided a transgenic non-human animal in which the cells of the non-human animal express the protein encoded by the nucleic acid construct according to any one of the previous aspects of the invention. The transgenic animal is preferably a mouse but may be another mammalian 20 species, for example another rodent, e.g. a rat or a guinea pig, or another species such as rabbit, or a canine or feline, or an ungulate species such as ovine, porcine, equine, caprine, bovine, or a non-mammalian animal species, e.g. an avian (such as poultry, e.g. chicken or turkey).

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It will be appreciated that the animals of the present invention may be engineered to comprise more than one reporter system according to the present invention.

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In embodiments of the invention relating to the preparation of a transfected host cell or a transgenic non-human animal comprising the use of a nucleic acid construct as previously described, the cell or non-human animal may be subjected to further transgenesis, in which the transgenesis is the introduction of an additional gene or

genes or protein-encoding nucleic acid sequence or sequences. The transgenesis may be transient or stable transfection of a cell or a cell line, an episomal expression system in a cell or a cell line, or preparation of a transgenic non-human animal by pronuclear microinjection, through recombination events in embryonic stem (ES) cells or by 5 transfection of a cell whose nucleus is to be used as a donor nucleus in a nuclear transfer cloning procedure.

Methods of preparing a transgenic cell or cell line, or a transgenic non human animal, in which the method comprises transient or stable transfection of a cell or a cell line, 10 expression of an episomal expression system in a cell or cell line, or pronuclear microinjection, infection of a cell or cell lines with a viral vector, recombination events in ES cells, or other cell line or by transfection of a cell line which may be differentiated down different developmental pathways and whose nucleus is to be used as the donor for nuclear transfer; wherein expression of an additional nucleic acid 15 sequence or construct is used to screen for transfection or transgenesis in accordance with any of the aspects of the invention. Examples include use of selectable markers conferring resistance to antibiotics added to the growth medium of cells, e.g. neomycin resistance marker conferring resistance to G418. Further examples involve detection using nucleic acid sequences that are of complementary sequence and which will 20 hybridise with, or a component of, the nucleic acid sequence in accordance with the first, second, third, or fourth aspects of the invention. Examples would include Southern blot analysis, northern blot analysis and PCR.

In an alternative embodiment of the present invention the host cell or transgenic 25 animal may also be engineered to comprise two or more constructs so as to allow a choice of readout or differentiable simultaneous readouts.

Preferably, the secreted/excreted reporter product or metabolite is a product that is 30 excreted in a body fluid of the transgenic animal. For example and without limitation in body fluids such as urine, saliva, tears, milk, cerebrospinal fluid and semen so that its presence is readily assayed and quantified in that fluid in advantageously a non-

invasive way. Alternatively the gene product may be assayable in serum, whole blood or tissue of the transgenic animal so that a gene activation event is detected after removal of serum, whole blood or tissue either *post mortem* or as a procedure during investigation in which case the transgenic animal is not sacrificed.

5

Preferably, the reporter gene encoding a reporter product or protein or molecule of the present invention possesses characteristics which favour urinary excretion of the reporter moiety.

10

Preferably, the reporter moiety is of relatively low molecular weight, typically in the region of < 120kDa and more preferably < 90kDa and more preferably still < 60kDa. Ideally the reporter moiety possesses a hydrophilic globular tertiary structure, has low bio-activity is stable in urine or the body fluid of choice and is clearly distinguishable from native molecules and is readily detectable and quantifiable.

15

We have found that SEAP is a suitable secreted/excreted reporter gene for the present invention, however it will be appreciated that other reporter moieties satisfying the above criteria will also be of utility in all the aspects of the present invention.

20

For example, other secreted/excreted molecules included in the present invention are selected from the group comprising; hormonal molecules, such as human chorionic gonadotrophin or FSH; antibodies such as γ and λ light chain (Bence Jones) proteins, in this particular embodiment of the invention a single chain may be excreted then recombined *ex vivo* with a partner chain whereby the combination is detectable only *ex vivo*; and enzymatic molecules such as feline urinary carboxylesterase.

25

According to a yet further aspect of the invention, there is provided the use of a nucleic acid construct in accordance with any one of the previous aspects of the invention for the detection of a gene activation event resulting from a change in altered metabolic status in a cell *in vitro* or *in vivo*.

30

The gene activation event may be the result of induction of toxicological stress, metabolic changes, disease that may or may not be the result of viral, bacterial, fungal or parasitic infection.

5 According to a yet further aspect of the invention there is provided the use of a nucleic acid construct comprising a nucleic acid sequence encoding a secreted/excreted protein, wherein said protein is heterologous to the cell in which it is expressed, for the detection of a gene activation event resulting from a change in altered metabolic status in a cell *in vitro* or *in vivo*.

10

The gene activation event may be the result of induction of toxicological stress, metabolic changes, disease that may or may not be the result of viral, bacterial, fungal or parasitic infection.

15

Uses in accordance with the various aspects of the invention also extend to the detection of disease states or characterisation of disease models in a cell, cell line or non human transgenic animal where a change in the gene expression profile within a target cell or tissue type is altered as a consequence of the disease. Diseases in the context of this aspect of the invention which are detectable under the methods

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disclosed may be defined as infectious disease, cancer, inflammatory disease, cardiovascular disease, metabolic disease, neurological disease and disease with a genetic basis.

25

An additional use in accordance with this aspect of the invention involves the growth of a transfected cell line in a suitable immunocompromised mouse strain (referred to as a xenograft), for example, the nude mouse, wherein an alteration in the expression of the reporter described in other aspects of the invention may be used as a measure of altered metabolic status of the host as a result of toxicological stress, metabolic changes, disease with a genetic basis or disease that may or may not be the result of viral, bacterial, fungal or parasitic infection. The scope of this use may also be of use

30

in monitoring the effects of exogenous chemicals or drugs on the expression of the reporter construct.

Aspects of the invention extend to methods of detecting a gene activation event *in* 5 *vitro* or *in vivo*.

In an alternative embodiment of the invention, the method comprises assaying a host cell stably transfected with a nucleic acid construct of the invention, or a transgenic non-human animal of the invention, in which the cell or animal is subjected to a gene 10 activation event that is signalled by expression of a peptide tagged secreted/excreted reporter gene.

In an alternative embodiment of the invention, the method comprises assaying a host cell stably transfected with at least one nucleic acid construct comprising a nucleic 15 acid sequence encoding a secreted/excreted protein, wherein said protein is heterologous to the cell in which it is expressed, or a transgenic non-human animal whose cells express such a construct, in which the cell or animal is subjected to a gene activation event that is signalled by expression of a peptide tagged reporter gene.

20 Accordingly there is provided a method of screening for, or monitoring of toxicologically induced stress in a cell or a cell line or a non-human animal, comprising the use of a cell, cell line or non human animal which has been transfected with or carries a nucleic acid construct as described above.

25 Toxicological stress may be defined as DNA damage, oxidative stress, hypoxia, post translational chemical modification of cellular proteins, chemical modification of cellular nucleic acids, apoptosis, cell cycle arrest, hyperplasia, immunological changes, effects consequent to changes in hormone levels or chemical modification of hormones, or other factors which could lead to cell damage.

The present invention advantageously is non-invasive since the reporter moiety is ultimately excreted without recourse to autopsy.

Accordingly, there is also provided a method for screening and characterising viral,
5 bacterial, fungal, and parasitic infection comprising the use of a cell, cell line or non human animal which has been transfected with or carries a nucleic acid construct as described above.

Accordingly, there is additionally provided a method for screening for cancer,
10 inflammatory disease, cardiovascular disease, metabolic disease, neurological disease and disease with a genetic basis comprising the use of a cell, cell line or non human animal which has been transfected with or carries a nucleic acid construct as described above.

15 In these contexts the cell may be transiently transfected, maintaining the nucleic acid construct as described above episomally and temporarily. Alternatively cells are stably transfected whereby the nucleic acid construct is permanently and stably integrated into the transfected cells' chromosomal DNA.

20 Also in this context transgenic animal is defined as a non human transgenic animal with the nucleic acid construct as defined above preferably integrated into its genomic DNA in all or some of its cells.

25 Expression of the peptide tagged, preferably epitope tagged secreted/excreted protein can be assayed for by measuring levels of the protein in cell culture medium or purified or partially purified fractions thereof.

30 Detection and quantification of the secreted/excreted proteins secreted from cultured cells into tissue culture medium or transgenic non-human animal body fluid may be achieved using a number of methods known to those skilled in the art. For example, immunological methods, such as ELISA or competitive ELISA, Western blot analysis

or fluorescence polarisation. Release of a labelled substrate e.g. radioactive (CAT) or fluorometric, colormetric. Detection of multiple substrates by for example mass spectrometry or, nuclear magnetic resonance (NMR).

5 In a further embodiment of the invention there is provided a method of detecting a reporter gene activation event comprising the steps of:

1. Transfecting a cell or microinjecting the pronucleus of a fertilised mouse egg with a nucleic acid sequence encoding a secreted/excreted protein optionally tagged with a peptide or protein as hereinbefore described and optionally using the microinjected egg or transfected mouse ES cell line;
- 10 2. Exposing the transfected cell, cell line or transgenic non human animal to a stimulus which may or may not cause a change in metabolic status resulting alteration in gene expression; and.
- 15 3. Using a suitable assay to determine the level expression of the secreted/excreted reporter protein which is optionally tagged, for example using detection methods such as ELISA, RIA, Mass spectrometry, NMR, telemetric methods.
- 20

In step (1), the detectable secreted/excreted protein may be a heterologous protein to the cell in which the nucleic acid construct is expressed. Such an "untagged" SEAP reporter protein may not therefore need a peptide or protein tag for detection.

25 Methods and uses in accordance with the present invention offer significant advances in investigating any area in which modified gene expression plays a significant role. Such reporter genes will be of use in cells and transgenic animals to detect activity of a variety of selected other genes. Specific applications include but are not restricted to:

1. Providing a rapid and robust *in vivo* screening system for assessing the potential toxic effects of chemicals.
2. Provide information on the mechanism of toxicity. Such information could be used to eliminate compounds from a selection process or suggest possible modifications to a compound.
3. Provide information on the effect of combinations of compounds.
4. Allow monitoring of variation in reporter gene expression over time by measuring levels of reporter(s) in urine at different time intervals.
5. Assessment of changes in gene expression associated with pathogenic infection.
6. Assessment of changes in gene expression associated with neurological, cardiovascular and metabolic diseases.
7. Assessment of changes in gene expression associated with cancer.
8. Provide information allowing validation of drug target selection e.g. by matching reporter expression profile to actions of toxins whose mechanism is defined and understood.
9. Use for evaluating compounds as therapeutic strategies aimed at reversing a toxic, metabolic, or degenerative phenotype.
10. Assessment of changes in gene expression resulting from environmental and/or behavioural changes.

The present invention will now be described with reference to the following examples which are present for the purposes of illustration only and should not be construed as being limited with respect to the invention. Reference in the application is also made to the following figures wherein:

FIGURE 1 illustrates the plasmid map for pCW2;

FIGURE 2 shows 3MC induction of pCW2 transiently-transfected into Hepa1 cells;

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FIGURE 3 shows 3MC induction of pCW2 stably-transfected into Hepa1 cells;

FIGURE 4 shows 3MC induction of Cyp1a1-SEAP activity detected in urine and blood of founder transgenic animals and;

FIGURE 5 shows 3MC induced SEAP activity in urine of transgenic animals.

5

Detailed Description of the Invention

Example 1

10 **Preparation of pCW2**

The sequence coding for 55.5kD secreted alkaline phosphatase (SEAP), a truncated form of placental alkaline phosphatase enzyme that lacks the GPI anchor, was excised from pSEAP2-Basic (Clonetech) by first converting a *Bsm*I restriction site by removing 3'-overhanging sequences and addition of *Bgl*II linker, then excision with *Bgl*II. The *Bgl*II SEAP fragment was inserted into a linearised pAHIR1 (Campbell et al J. Cell Sci. 109, 2619-2625 (1996)) thereby placing this reporter gene downstream of 8.5kb of 5'-flanking sequences, the first exon and intron, and the second exon to +2548 of the rat Cyp1A1 promoter. Figure 1 shows the plasmid map for pCW2.

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Example 2

3MC Induced Cyp1A1-SEAP Vector Expression in Transfected Cells.

Hepa-C1C7 cells (cultured in DMEM supplemented with 10%FCS, 2mM l-glutamine, at 5%CO₂) were transfected with pCW2 either transiently or stably (in conjunction with pSVNeo) using the calcium phosphate co-precipitation method. Briefly, 5µg of plasmid (+ 1µg pSVNeo for stable transfections) was mixed with calcium chloride and HEPES buffered saline to form a calcium phosphate-DNA precipitate which was left incubating with the cells for 5 hours. The medium was then replaced with fresh growth medium or with selection medium (growth medium supplemented with 400µg/ml G418, for stable transfections). Transiently transfected cells were plated into 6 well plates and were incubated with increasing doses of 3MC dissolved in culture medium. For stable transfections, once individual colonies could be identified on the plates the

colonies were pooled and incubated with 2 μ g/ml 3MC. Forty eight hours after induction with 3MC the medium was assayed for SEAP activity.

SEAP activity was determined using the "SEAP Reporter Gene Assay, chemiluminescent" (Roche). Human placental alkaline phosphatase from the kit or from Sigma was used as a positive control. Briefly, samples and standards (0.8pg - 8 μ g/ml) were diluted in dilution buffer and heated to 65°C for 30 minutes. After centrifugation to remove precipitated material the samples were placed on ice and then pipetted into a black 96 well plate (Nunc) together with inactivation buffer for 5 minutes incubation. The activity of the SEAP in each sample was then revealed after a 10 min incubation of the substrate and reading the light emitted with a luminometer (EG&G Berthold 96V microplate luminometer). FIGURE 2 shows 3MC induction of pCW2 transiently-transfected into Hepal cells and FIGURE 3 shows 3MC induction of pCW2 stably-transfected into Hepal cells.

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Example 3

Generation of Transgenic Mice with pCW2.

A 10kb *NotI* restriction fragment from pCW2 containing the Cyp1A1 promoter, SEAP and polyadenylation sequences was purified by gel electrophoresis, to remove plasmid sequences, and injected into the male pronucleus of fertilized mouse eggs (F1 C57/BL6 x CBA) at a concentration of 1.5ng/ μ l. Injected eggs that survived culturing to the two-cell stage were transferred to pseudopregnant females (F1) that were allowed to come to term. Genotyping for transgenic status was done by polymerase chain reaction (PCR) on DNA extracted from tail biopsy at 4-6 weeks of age (Whitelaw et al. Transgenic Res 1, 3-13 (1991)) using forward primer 5'-CGCCAAGAACCTCATCATCT-3' (SEQ ID NO:1) and reverse primer 5'-CGTCAAT GTCCATGTTGGAG-3' (SEQ ID NO:2) recognising SEAP cDNA sequences.

30 In one study from 683 eggs injected, 127 pups were born. Twelve (2% of 683) of these pups were identified as transgenic by PCR; 7 females and 5 males.

Example 4**3MC-Induced Cyp1A1-SEAP Vector Expression in Transgenic Mice.**

To demonstrate induction for Cyp1A1-SEAP expression *in vivo*, mice were treated with 3-methylcholanthrene (3MC). Induction followed procedures evaluated previously for rat Cyp1A1-LacZ transgenic mice (Campbell et al J. Cell Sci. 109, 2619-2625 (1996)). 3MC was administered to female and male mice (of at least 8 weeks of age) as a suspension in Mazola brand corn oil by intraperitoneal injection. Test animals were either transgenic or non-transgenic age-matched animals with the same genotype were i.p. injected once every 24 hours with 40mg/kg 3-methylcholanthrene (3MC) in maize oil for three consecutive injections. Control animals were injected with an equal volume of carrier corn oil only. All animals were killed by cervical dislocation 24 hours following the final dose.

Samples of the liver and kidney were removed, washed once in phosphate buffered saline and then homogenised in HB buffer (140mM NaCl, 50mM Tris-HCl pH 7.5, 1mM EDTA, 1% w/v Triton X-100) using a dounce glass homogeniser until a smooth solution was formed. Insoluble proteins were removed by centrifugation at 13000 rpm for 5 minutes and the cleared supernatant was assayed for protein content using the Pierce protein determination kit. For each tissue a final concentration of 0.8mg/ml was used in the SEAP assay.

Urine and blood samples were centrifuged at 5000 rpm for 5 minutes to remove any solid waste (from the urine) or coagulated cells (from the blood) and were assayed for SEAP activity immediately. Urine was used undiluted in the assay, whereas blood samples were diluted 1:100 and 1:500 with distilled water prior to the SEAP assay. SEAP activity was assayed as described in example 2. FIGURE 4 shows 3MC induction of Cyp1a1-SEAP activity detected in urine and blood of 3 out of 5 of founder transgenic mice analysed and FIGURE 5 shows 3MC induced SEAP activity detected in the urine of transgenic mice.

Claims

1. A nucleic acid construct comprising a nucleic acid sequence comprising a reporter gene encoding a reporter protein that is secretable as a protein or product from a cell where it is expressed or produced and that is excreted from a whole animal.
2. A nucleic acid construct according to claim 1 wherein the secretable/excreted protein or product is produced by modulated gene transcription.
- 10 3. A nucleic acid construct according to claim 1 wherein the secretable/excreted protein or product is produced by increased reporter translation.
4. A nucleic acid construct according to claim 3 wherein the increased reporter translation is as a result of increased stability or decreased turnover of mRNA.
- 15 5. A nucleic acid construct according to claim 1 wherein the secretable/excreted protein or product is produced by post-translational modulation.
6. A nucleic acid construct according to claim 5 wherein the post-translational modulation is increased reporter stability through removal of polyubiquination or as the result of accumulation or excretion of small molecule metabolites
- 20 7. A nucleic acid construct according to any preceding claim further comprising a peptide tag optionally in the form of an epitope tag.
- 25 8. A nucleic acid construct according to any preceding claims additionally comprising a promoter element upstream of the (i) a nucleic acid sequence encoding a secreted/excreted protein, and/or (ii) a nucleic acid sequence encoding a peptide tag.
- 30 9. A nucleic acid construct according to any preceding claim wherein the secreted/excreted reporter protein is SEAP.

10. A nucleic acid construct according to any preceding claim wherein the secreted/excreted reporter protein/product is selected from the group comprising hormonal molecules optionally in the form of human chorionic gonadotrophin or FSH; antibodies optionally in the form of γ and λ light chain (Bence Jones) proteins, and enzymatic molecules optionally in the form of feline urinary carboxylesterase.
5
11. A host cell transfected with at least one nucleic acid construct according to any one of claims 1 to 10.
- 10 12. A transgenic non-human animal in which the cells of the non-human animal express the protein encoded by the nucleic acid construct according to any one of claims 1 to 10.
- 15 13. A transgenic non-human animal according to claim 12, in which the non-human animal is a mammal.
14. A transgenic non-human mammal according to claim 13, in which the mammal is a mouse.
- 20 15. A transgenic non-human animal according to any one of claims 12 to 14 wherein the secreted/excreted reporter product or protein or molecule is excreted in a body fluid selected from the group comprising urine, saliva, tears, milk, cerebrospinal fluid and semen.
- 25 16. A transgenic non-human animal according to any one of claims 12 to 15 wherein the secreted/excreted reporter product or protein or molecule is excreted in urine.

17. A host cell according to claim 11 or a transgenic non-human animal according to any one of claims 12 to 16 wherein the secreted/excreted reporter moiety is of relatively low molecular weight, in the region of < 60-120kDa.

5 18. A host cell according to claim 11 or a transgenic non-human animal according to any one of claims 12 to 16 wherein the secreted/excreted reporter moiety possesses a hydrophilic globular tertiary structure, has low bio-activity is and is clearly distinguishable from native molecules so that it is readily detectable and quantifiable.

10 19. A host cell according to claim 11 or a transgenic non-human animal according to any one of claims 12 to 16 comprising more than one nucleic acid construct according to claims 1 to 10.

15 20. Use of a nucleic acid construct according to any one of claims 1 to 10 for the detection of a gene activation event resulting from a change in altered metabolic status in a cell *in vitro* or *in vivo*.

20 21. Use according to claim 20, in which the gene activation event is the induction of toxicological stress, metabolic changes, or viral, bacterial, fungal or parasitic infection.

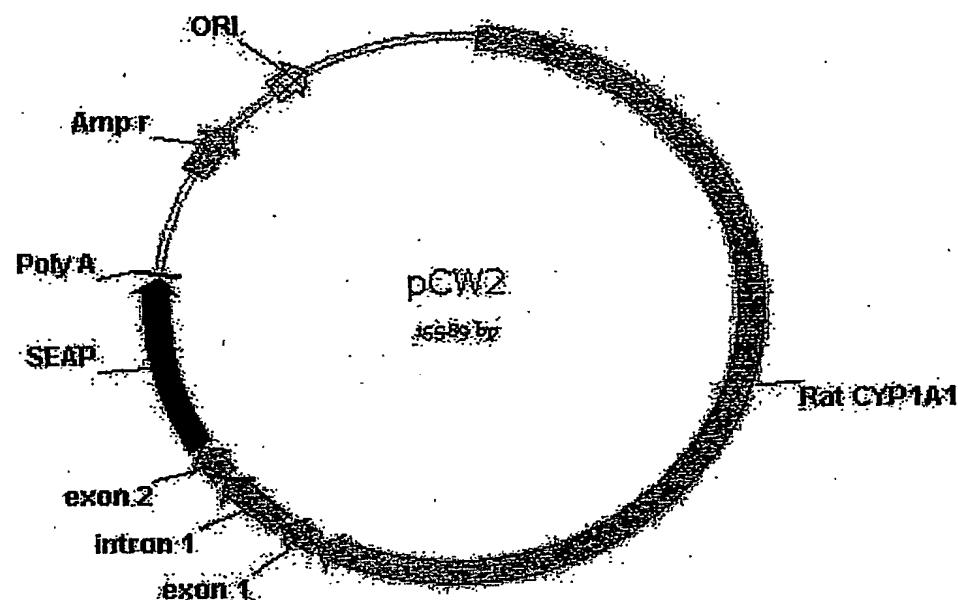
25 22. A method of detecting a gene activation event in a cell *in vitro* or *in vivo*, comprising assaying a host cell stably transfected with a nucleic acid construct in accordance with any one of claims 1 to 10, or a transgenic non-human animal according to any one of claims 12 to 16, in which the cell or animal is subjected to a gene activation event that is signalled by expression of a secreted/excreted reporter protein optionally the protein being tagged with an epitope.

30 23. A method of screening for, or monitoring of, toxicologically induced stress in a cell or a cell line or a non-human animal, comprising the use of a cell, cell line or non

human animal which has been transfected with or carries a nucleic acid construct according to any one of claims 1 to 10.

24. A method for screening and characterising viral, bacterial, fungal, and parasitic infection or for screening for cancer, inflammatory disease, cardiovascular disease, metabolic disease, neurological disease and disease with a genetic basis comprising the use of a cell, cell line or non human animal which has been transfected with or carries a nucleic acid construct according to any one of claims 1 to 10.

FIGURE 1



5

FIGURE 2

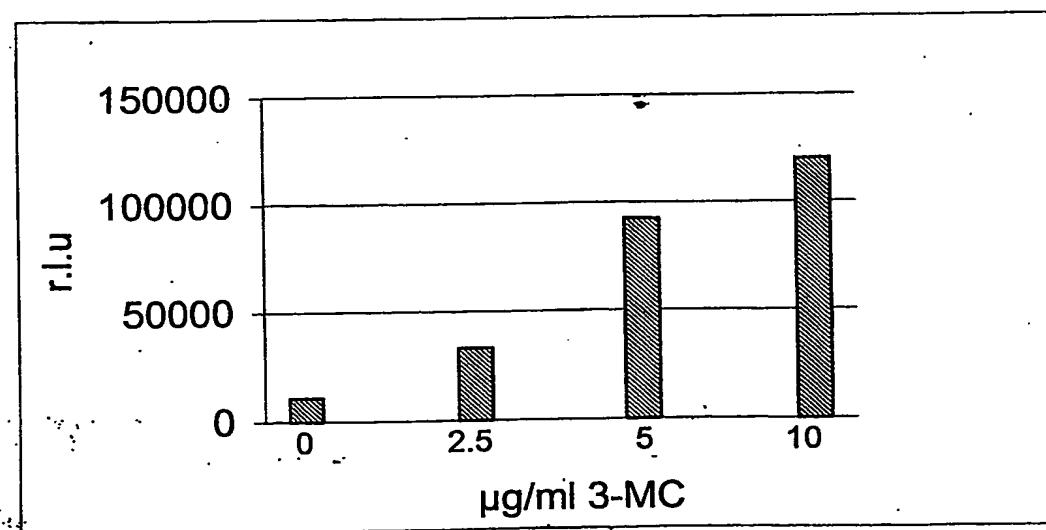
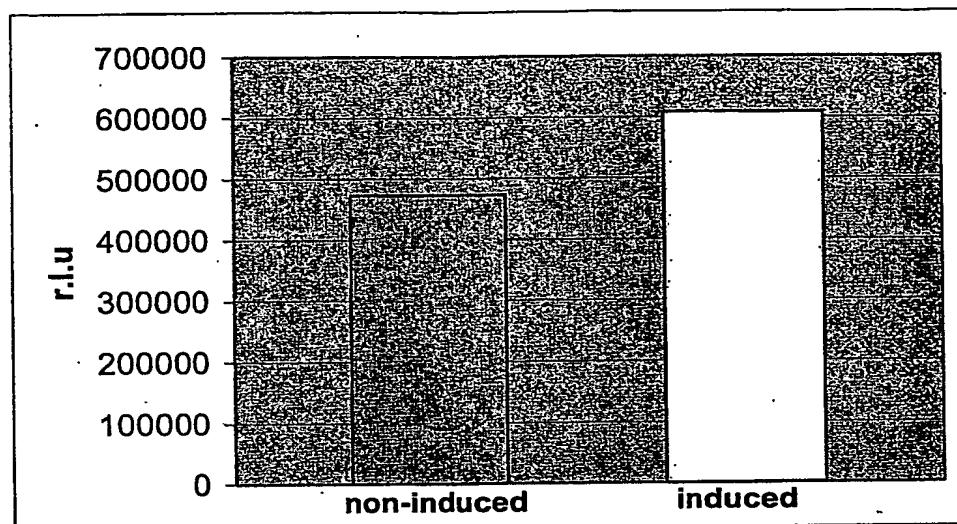


FIGURE 3



5 FIGURE 4

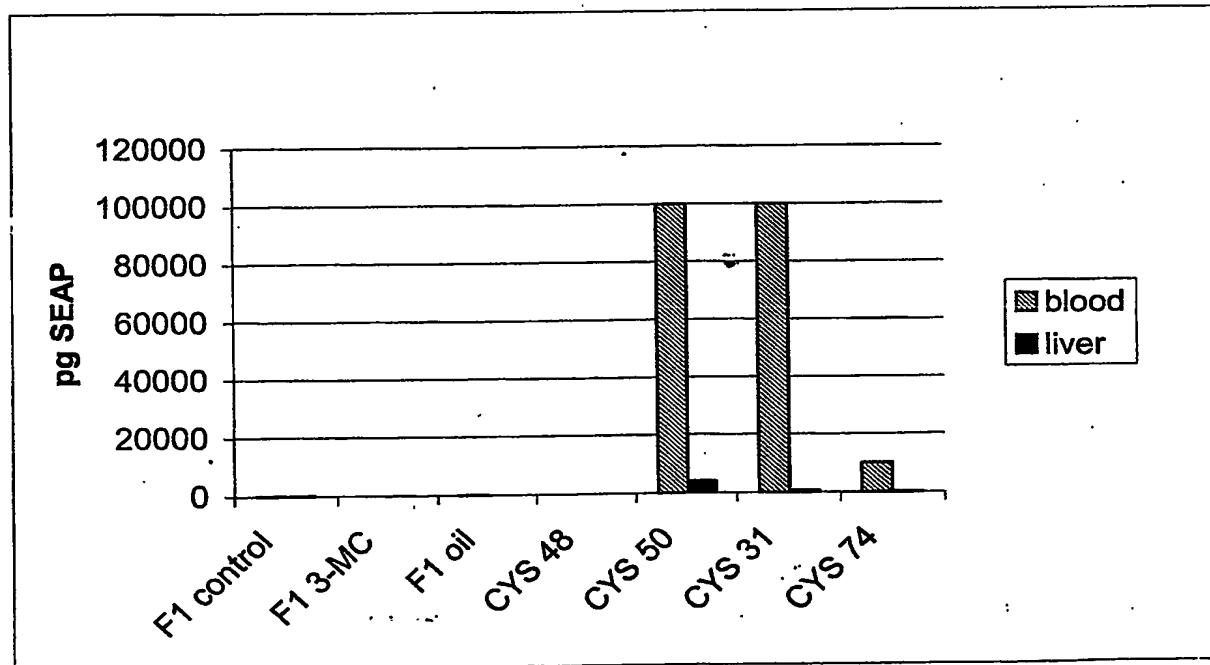
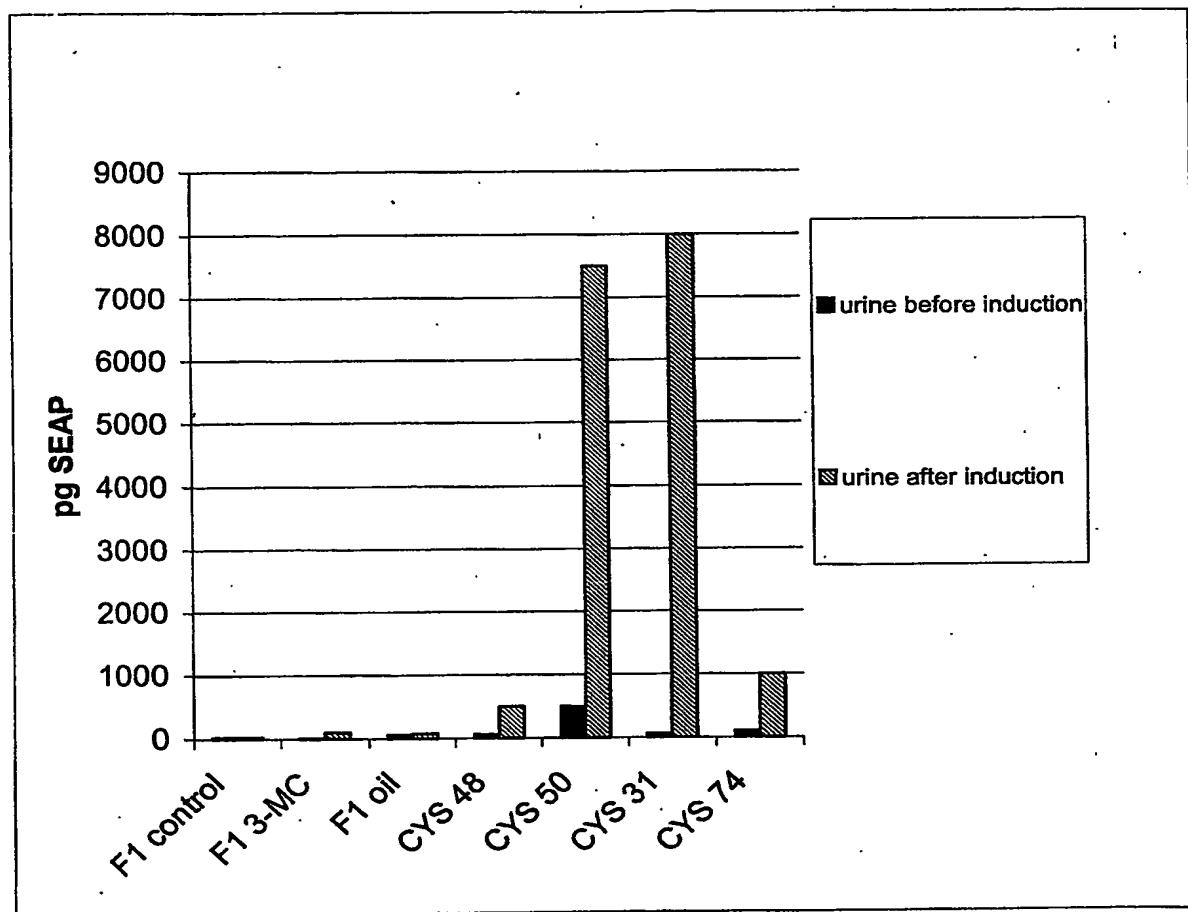


FIGURE 5



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